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(54) Title: DETECTION OF HYPOXIA (57) Abstract Novel nitroaromatic compounds and immunogenic conjugates comprising a novel nitroaromatic compound and a carrier protein are disclosed. The invention further presents monoclonal antibodies highly specific for the claimed nitroaromatic compounds, the compounds' protein conjugates, the compounds' reductive by-products, and adducts formed between the compounds and mammalian hypoxic cell tissue proteins. The invention is further directed to methods for detecting tissue hypoxia using immunohistological techniques, non-invasive nuclear medicinal methods, or nuclear magnetic resonance. Diagnostic kits useful in practicing the methods of claimed invention are also provided.		

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DETECTION OF HYPOXIA

FIELD OF THE INVENTION

This invention generally relates to a class of nitroaromatic compounds that, when activated by reductive metabolism, bind to hypoxic cells. This reductive metabolism and binding increase as the oxygen concentration of cells decreases, which enables these compounds to be used as indicators of hypoxia. The present invention presents novel nitroaromatic compounds; immunogenic conjugates comprising the novel nitroaromatic compounds and proteins; and monoclonal antibodies specific for the novel nitroaromatic compounds of the invention, their protein conjugates, their reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention. The invention is further directed to methods for detecting levels of low oxygen in tissue. Detection may be done directly using methods such as imaging techniques involving specific isotopes attached to the nitroaromatic drug, or indirectly using the monoclonal antibodies (mAbs) in immunohistological assays. Still further, the present invention is directed to kits for performing the methods of the invention.

BACKGROUND OF THE INVENTION

One of the most important goals in oncology is the identification and elimination of treatment resistant cells; hypoxic cells are the most familiar examples of this type of cell. Kennedy, et al., *Biochem. Pharm.* 1980, 29, 1; Moulder, et al., *Int. J. Radioat. Oncol. Biol. Phys.* 1984, 10, 695;

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Adams, *Cancer*, 1981, 48, 696. Hypoxic cells are seldom found in normal tissues, and are generally found only in conjunction with certain tumors, vascular diseases, or after a stroke.

5 As certain tumors enlarge, the tissue often outgrows its oxygen and nutrient supply because of an inadequate network of functioning blood vessels and capillaries. Although the cells deprived of oxygen and nutrients may ultimately die, at any given time a tumor may
10 produce viable hypoxic cells. These hypoxic cells, although alive, have very low oxygen concentrations because of their remoteness from the blood vessels.

 The level of molecular oxygen has important implications in disease diagnosis and prognosis. In
15 radiation oncology, for example, hypoxic cells in solid tumors are highly resistant to killing by radiation and some forms of chemotherapy. When chemotherapeutic agents are administered to patients the agents are carried through the functioning blood vessels and capillaries to the target
20 tissue. Because hypoxic tissue lacks a fully functioning blood supply network, the chemotherapeutic drugs may never reach the hypoxic cells; instead, intervening cells scavenge the drug. The result is that the hypoxic cells survive and recurrence of the tumor is possible. Kennedy, et al., *supra*.

25 Tissue hypoxia also hinders the effectiveness of radiation therapy, especially of neoplasms. Radiation treatment is most effective in destroying oxygen containing cells because oxygen is an excellent radiation sensitizer.
30 The presence of hypoxic cells impedes this treatment because their low oxygen concentration renders the ionizing radiation relatively ineffective in killing the cancerous cells. Therefore, hypoxic cells are more likely to survive radiation therapy and eventually lead to the reappearance of the tumor.
35 The importance of hypoxic cells in limiting radiation responsiveness in animal tumors is well known, Adams, *supra*; Moulder, et al., *supra*; Chapman, et al., "The Fraction of

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Hypoxic Clonogenic Cells in Tumor Populations," in Biological Bases and Clinical Implications of Tumor Radioresistance 61, G.H. Fletcher, C. Nevil, & H.R. Withers, eds., 1983. Studies have revealed that such resistant cells greatly affect the ability of radiation and chemotherapy to successfully sterilize tumors in animals. Substantial work since that time has shown similar problems in human tumors. Despite the progress in animal studies regarding the identification of hypoxic cells, limited success has been achieved in humans. One reason for this disparity may relate to differences in tumor growth and other host related factors, but in addition, there has been no suitably accurate method to assess tissue oxygen at a sufficiently fine resolution.

Venous oxygen pressure is generally ~35 Torr, an oxygen level providing nearly full radiation sensitivity. As the oxygen level decreases below 35 Torr, radiation resistance gradually increases, with half-maximal resistance at about 3.5 Torr, and full resistance at about 0.35 Torr. Therefore, it is necessary to measure much lower oxygen levels than are usually encountered in normal tissue. Current technology does not meet this need. Oxygen partial pressure measured using current techniques often yields an average value for large numbers of neighboring cells. This is a severe impediment for detection and diagnosis because histological evaluation of solid tumors suggest that important changes in cellular oxygen can occur over dimensions of even a few cell diameters. Urtasun, et al., *Br. J. Cancer*, 1986, 54, 453.

Nitroheterocyclic drugs have been under extensive investigation as oxygen indicators. It is known that this class of compounds has the potential for resolution at the cellular level and can provide sufficient sensitivity to monitor the low oxygen partial pressures described above. This technique involves the administration of nitroaromatic drugs to the tissue of interest. The drugs undergo bioreductive metabolism at a rate which increases substantially as the tissue's oxygen partial pressure

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decreases. The result of this bioreductive metabolism is that reactive drug products are formed which combine chemically to form adducts with predominantly cellular proteins. Because the metabolic binding of these compounds to cellular macromolecules is inhibited by oxygen, these compounds bind to hypoxic cells in preference to normal, healthy, oxygen-rich tissue. This preferential metabolic binding, or adduct formation, provides a measure of the degree of hypoxia. Koch, et al., *Int. J. Radiation Oncology Biol. Phys.*, 1984, 10, 1327.

Misonidazole, MISO 3-methoxy-1-(2-nitroimidazol-1-yl)-2-propanol, and certain of its derivatives have been under extensive investigation as indicators of hypoxia in mammalian tissue. Chapman, et al., *Int. J. Radiat. Oncol. Biol. Phys.*, 1989, 16, 911; Taylor, et al., *Cancer Res.*, 1978, 38, 2745; Varghese, et al., *Cancer Res.*, 1980, 40, 2165. The ability of certain misonidazole derivatives to form adducts with cellular macromolecules, referred to as binding throughout this application, has formed the basis of various detection methods.

For example, H³ or C¹⁴ labelled misonidazole has been used *in vitro* and *in vivo*, with binding analyzed by liquid scintillation counting or autoradiography. Chapman, 1984 *supra*; Urtasun, 1986, *supra*; Franko, et al., *Cancer Res.*, 1987, 47, 5367. A monofluorinated derivative of misonidazole has utilized the positron emitting isotope F¹⁸ for imaging bound drug *in vivo*, Rasey, et al., *Radiat. Res.*, 1987, 111, 292. A hexafluorinated derivative of misonidazole has been assayed directly (no radioactive isotopes) via nuclear magnetic resonance spectroscopy (NMR or MRI) techniques. Raleigh, et al., *Int. J. Radiat. Oncol. Biol. Phys.*, 1984, 10, 1337. Polyclonal antibodies to this same derivative have allowed immunohistochemical identification of drug adducts. Raleigh, et al., *Br. J. Cancer*, 1987, 56, 395. An iodine isotope has been incorporated into another azomycin derivative, azomycin arabinoside, allowing radiology

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techniques of detection. Parliament, et al., *Br. J. Cancer*, 1992, 65, 90.

A fluorescence immunohistochemical assay for detecting hypoxia is described in the literature. Raleigh, et al., 1987, *supra*. A method for preparing immunogenic conjugates for use in such assays is broadly disclosed in U.S. Patent No. 5,086,068, issued to Raleigh, et al., on February 4, 1992 ("Raleigh patent"). The Raleigh patent describes a method for preparing an immunogenic conjugate comprising a known fluorinated misonidazole derivative and an immunogenic carrier protein, hemocyanin. The resulting conjugate is used to raise rabbit polyclonal antibodies specific for the misonidazole derivative. Fluorescence immunohistochemical studies showed that the polyclonal antibodies bound to hypoxic (central) regions of spheroids (a multicellular aggregate of cells in tissue culture having some properties more closely related to tumors) and tumor sections in patterns similar to those revealed by audioradiographic studies using radioactive drug alone, i.e. without polyclonal antibodies. The misonidazole derivative used in this method was the hexafluorinated 2-nitroimidazole (CCI-103F) described above in connection with the NMR studies, 1-(2-hydroxy-3-hexafluoroisopropoxypropyl)-2-nitroimidazole.

The Raleigh patent's technology, of conjugating a small antigen to a large carrier protein to elicit an immune response, is a central basis of antibody production and is well known in the art. Those skilled in the art would also appreciate that nitroaromatics must be activated by chemical or biochemical reduction to cause adducts to form with cellular macromolecules. Further, it has not been possible to produce monoclonal antibodies using the methods described in the Raleigh patent and paper (Raleigh et al., 1987, *supra*).

The bioreductive drug assays described above do not directly measure oxygen partial pressure, even though this is the required value, using the example of radiation therapy to

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predict radiation response. Rather, the assays measure adduct formation, a biochemical process which is inhibited by oxygen. The data generated using these methods has shown that the degree of inhibition by oxygen varies substantially from tissue to tissue. Franko, et al., 1987, *supra*. Furthermore, the maximum rate of adduct formation in the complete absence of oxygen is also highly variable from tissue to tissue, as is the maximum percentage of inhibition by oxygen, Koch, in *Selective Activation of Drugs by Redox Processes*, Plenum Press, pp. 237-247, Adams, et al., eds, New York, 1990. Another way of expressing these limitations is that the bio-reductive formation of nitroaromatics provide only a relative indication of varying oxygen levels, but is inadequate at providing an absolute measurement of oxygen partial pressure because there are several factors which affect adduct formation in addition to changes in oxygen, non-oxygen-dependent factors. Additionally, the choice of nitroaromatic drug affects the variability related to the non-oxygen-dependent factors.

Most research has focused on misonidazole and certain of its derivatives. However, misonidazole is the most susceptible of several drugs tested to non-oxygen-dependent variations in adduct formation. Koch, *Selective Activation, supra*. Other problems relate to various physicochemical properties of existing drugs, all of which can influence the non-oxygen dependent variations in adduct formation. For example, the hexafluorinated misonidazole derivative described above had a high degree of insolubility.

Although radiochemical tracers provide a sensitive method for detecting tissue hypoxia, the biohazards and costs associated with these techniques are a significant drawback. The amount of radioactivity associated with the administration of such labelled drugs, which still requires a tissue biopsy, becomes a substantial problem in animal studies and an even greater problem in humans where 30 millicuries of tritiated drug are typically used. Urtasun, et al., 1986, *supra*. C^{14} is prohibitively expensive and

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causes unacceptable radiation exposures. The use of radioactive tracers is generally not acceptable because of the stringent requirements associated with handling radioactive tissues and bodily fluids. There are also practical limitations to the use of radioactive tracers. For example, the delay required for audioradiographic analysis of the tissue sections, often several weeks, is a very serious impediment to the rapid analysis required in treatment determination. Moreover, toxicity problems associated with certain misonidazole derivatives resulted in the drug being administered at a relatively low concentration, which decreased detection sensitivity.

The Raleigh patent discloses immunogenic conjugates useful for producing polyclonal antibodies, but data generated using the patent's teachings has produced variable results, problematic in a detection technique. Furthermore, independent experimentation performed according to the Raleigh patent's methods did not reproduce the high degree of conjugation between the misonidazole derivatives and the protein as was claimed.

There is no method currently available that can safely and consistently assay the oxygen level in mammalian tissue. There has been a long felt need for safer and more predictable oxygen detection methods without the concomitant hazards associated with radioactivity. The present invention addresses this need among others.

SUMMARY OF THE INVENTION

This invention presents novel nitroaromatic compounds; immunogenic conjugates comprising the novel nitroaromatic compounds and proteins; and monoclonal antibodies specific for the novel nitroaromatic compounds of the invention, their protein conjugates, their reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention. The novel compound's protein conjugates, reductive byproducts, and adducts formed between mammalian

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hypoxic cells and the compounds of the invention may be generally referred to as compositions throughout this application. The novel compounds and compositions of the invention, and the methods according to this invention,
5 provide the basis for sensitive and precise methods for detecting tissue hypoxia.

The compounds of the invention possess unique properties that make them safer and more predictable oxygen indicators than previous compounds. The structure of the
10 parent 2-nitroimidazole, etanidazole, N-(2-hydroxyethyl)-2(2-nitro-1H-imidazol-1-yl)acetamide, has been shown to be less susceptible to non-oxygen-dependent variations in adduct formation than is misonidazole.

Preferred embodiments of the invention containing 5
15 fluorines have now been provided which maintain high solubility. A greatly preferred embodiment is 2(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide, referred to as EF5 throughout this application. This increased solubility over misonidazole derivatives currently
20 in use permits administering a higher drug concentration resulting in enhanced detection sensitivity without the toxicity observed with current methods.

Furthermore, the formulation of specific drug-protein conjugates has allowed the formation of monoclonal
25 antibodies against such conjugates produced by cellular bioreductive metabolism. Despite world-wide efforts, monoclonal antibodies have not previously been prepared against any nitroaromatic drug adducts, and it is essential to have a reliable and consistent source of such monoclonal
30 antibodies for any clinical assay. The development of monoclonal antibodies represents a significant advance in technology because of the biohazards associated with the use of radioactive tracers such as H^3 and C^{14} .

In contrast to the EF5's overall solubility, is the
35 hydrophobicity of its halogenated side chain. This characteristic of the compounds of the invention improves detection efficiency because the side chain's hydrophobic

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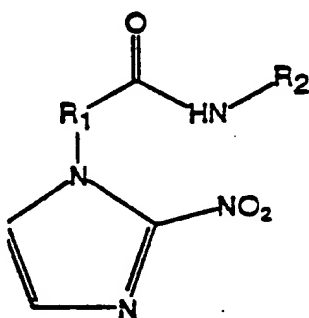
nature causes a greater proportion of macromolecular adducts to form between the drug-protein conjugate and the hypoxic cell proteins than would be the case for more hydrophilic derivatives. The relatively large number of fluorines
5 enhances any assay of this atom, with F^{19} preferred for magnetic resonance imaging (MRI) and F^{18} for positron emission tomography (PET). These competing properties are unique to the compounds of the invention and add to the novelty and utility of the invention.

10 Further, the side chain of the present compounds is highly non-physiological, which is believed to contribute to their antigenic characteristics. The monoclonal antibodies of this invention are highly specific for the novel
15 nitroaromatic compounds of the invention, their protein conjugates, their reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention. This specificity makes these antibodies superior detectors than the polyclonal antibodies currently used in
20 the art. As indicated above, a consistent source of identical antibodies is required for clinical assays. The novel compounds of the invention provide the basis for a sensitive, versatile, and more accurate method for detecting tissue hypoxia.

Thus, the present invention presents a novel class
25 of compounds, similar in core structure to misonidazole but having new side chains that make them much more predictable oxygen indicators and much more amenable to immunohistochemical and other assays. The novel compounds and compositions of the invention and the corresponding
30 methodologies provide techniques for measuring the degree of hypoxia in mammalian tumors with a precision and sensitivity that has not been achieved before. These novel compounds and compositions may be used to detect hypoxia using standard
35 nuclear medical procedures with a consistency not previously observed in the art. These novel compounds also provide the basis for immunological assays.

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The novel class of compounds of this invention are halogenated 2-nitroimidazole derivatives capable of stimulating the immunogenic process and enabling the production of monoclonal antibodies specific for the compounds of the invention, their protein conjugates, reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention. The compounds of the present invention have the general structure depicted below



wherein R_1 may be either CH_2 , CHX , or CX_2 , where X is a halogen atom. The R_2 substituent is an alkyl group containing 1 to 6 carbons, and preferably having up to about 6 halogen atoms. The preferred halogen atom is fluorine. In accordance with preferred embodiments, compounds of the invention will have about 5 fluorine atoms, R_1 will be CH_2 and R_2 will be $CH_2CF_2CF_3$. The invention further presents compositions comprising the novel compounds of the invention bound to a protein. These drug-protein conjugates are capable of eliciting an immune response that enables the production of monoclonal antibodies highly specific for the compounds of the invention; their reductive byproducts, protein conjugates, and adducts formed between tissue proteins and the compounds of the invention. The monoclonal antibodies' specificity for the claimed compounds and compositions provides the basis for sensitive, accurate, and precise immunohistochemistry-assay-based detection of hypoxia. In a preferred embodiment of the invention, the protein carrier may be albumin, Bowman Birk inhibitor, or lysozyme. In another preferred embodiment, the protein is lysozyme, R_1 is CH_2 , and R_2 is $CH_2CF_2CF_3$. In a more preferred

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embodiment, the protein is Bowman Birk inhibitor (BBI), R_1 is CH_2 , and R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$.

The invention is further directed to pharmaceutical formulations of the novel drug compounds. In accordance with
5 preferred embodiments, a compound of the invention is dissolved or dispersed in a pharmaceutically acceptable diluent. Preferred diluents are non-pyrogenic physiological saline.

The invention is also directed to formulations of
10 immunogenic conjugates comprising the novel drug compounds of the invention bound to a protein carrier and dissolved or dispersed in a diluent.

In another aspect of the invention, monoclonal antibodies specific for the compounds of the invention, their
15 protein conjugates, reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention are provided. The protein conjugates, reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention are referred to
20 generally as compositions. In a preferred embodiment of the invention, monoclonal antibodies will be specific for compounds and compositions of the invention where the halogen atom is fluorine. In a more preferred embodiment of the invention, the monoclonal antibodies will be specific for
25 compounds and compositions having about 5 fluorine atoms. In yet a more preferred embodiment, the monoclonal antibodies will be specific for compounds and compositions of the invention where R_1 is CH_2 . In another preferred embodiment, the monoclonal antibodies of the invention will be specific
30 for the compounds and compositions of the invention where R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$. In a still more preferred embodiment, the monoclonal antibodies will be specific for compounds and compositions where R_1 is CH_2 and R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$. Methods for preparing the monoclonal antibodies are also provided. As
35 will be appreciated, the monoclonal antibodies of the invention can be either to the novel compounds per se or to the compounds bound to a protein.

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In a further aspect of the invention, methods for assaying tissue hypoxia are provided. A tissue sample is assayed using immunohistochemical techniques or imaging techniques. Imaging techniques may be used for non-invasive analysis. Imaging methods comprise using the novel compounds of the invention without immunohistochemical assays, and preferably without the use of monoclonal antibodies to detect hypoxic cells. The mammal is administered a compound of the invention, dissolved or dispersed in a suitable pharmaceutical carrier or diluent, as discussed above. The compound is allowed to clear from the mammal, and then a portion of the mammal containing the tissue of interest is analyzed non-invasively such as through magnetic resonance imaging (MRI) or positron emission tomography (PET). A proportion of the compound will remain in the body, bound or associated with hypoxic cells. In the case of MRI, conventional non-radioactive (F^{19}) isotopes of fluorine are used. In the case of PET, a compound of the invention must first be formulated with the positron emitting isotope F^{18} .

In another preferred embodiment of the invention, the assay methods use immunochemistry. These methods generally comprise administering to a mammal, as above, a compound of the invention; obtaining a tissue sample; and detecting the presence of adducts formed between cells of the sample and a compound of the invention by contacting the tissue sample with the invention's monoclonal antibodies associated with a detection system. The mAb will be specific for the adduct; that is, the mAb will be specific for the adduct formed between tissue proteins and the compound previously administered. The degree of tissue hypoxia is determined by quantifying the level of antibody interaction with the cells such as by using enzyme linked immunosorbant assay (ELISA), microdialysis, immunohistochemical staining, or other immunological protocols.

Yet another embodiment of the invention requires that the drug-hypoxic tissue adducts be detected *in situ*. A compound of the invention is administered to a mammal and

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allowed to clear from the non-binding tissue. A labelled mAb specific for the adduct; that is, an mAb specific for an adduct formed between tissue proteins and the drug previously administered, is administered. The antibody is labelled with isotopes suitable for radiographic or nuclear medicine assays and will bind to the adducts formed with the hypoxic tissues. The advantages of this embodiment are that the antibodies can be used in a non-invasive assay of the tissue, there being more flexibility in the labelling of the antibody than in the labelling of the 2-nitroimidazole drug, whose structure is relatively fixed.

Kits useful for diagnostic applications comprising the novel compounds or compositions are also within the ambit of the present invention. These kits include a drug formulation of the invention, along with immunochemical reagents, especially including monoclonal antibodies of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a compound of the invention binding to cells in a manner which increases substantially as the oxygen concentration decreases. Binding was detected using a radioactive form of a specific compound of the invention, EF5. The graph symbols are defined as follows: circles represent hypoxic cells; squares represent cells containing an intermediate level of oxygen (~3 Torr); and triangles represent cells containing a higher level of oxygen (15 Torr). In each case the solid symbols are for trichloroacetic acid (TCA) precipitable adducts, and the open symbols are for TCA soluble adducts. The cells in this experiment were 9L glioma cells.

Figure 2 is a graphical representation of the specificity of a mAb of the invention against several antigens. A mAb of the invention is at least 100 times more reactive toward EF5, than other, similar nitroaromatic drugs. The graph symbols are defined as follows: solid circles represent EF5; open circles represent etanidazole (ETAN);

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open triangles represent MISO; closed squares represent CCI-103F; crosses represent NP-6 (2,4-dinitro-1H-pyrrole-2-ethanol); and open squares are dimethylmisonidazole, 3-methoxy-1-[2-Nitro-4,5-dimethylimidazol-1-yl]-2-propanol (DMM).

Figure 3 is a graphical representation of the specificity of a mAb of the invention in several different forms, against a compound of the invention, EF5, adducted to BBI. The mAb was chemically modified to add a fluorescent moiety, amino methyl coumarin acetic acid (AMCA) using two different coupling chemistries. Fluorescent dyes may be conjugated onto mAbs of the invention without affecting the mAbs' activity. The graph symbols are defined as follows: open circles represent the monoclonal antibodies' specificity without the presence of the detection moiety, AMCA; closed triangles represent the coupling of the dye to an N-hydroxysulfocuccinimide group (NHS sulfo Hi), which then reacts with primary amines of the protein of interest; closed squares represent similar methods where the reactive group is a pyridyldithiopropionamide (HPDP hi), which reacts with free thiol groups on the protein of interest. The thiols were generated using mild radiochemical reduction methods (see Example 1) to reduce a small portion of the antibody's disulfide residues.

Figure 4 depicts the binding, using ELISA based techniques, of a mAb of the invention to proteins derived from EMT-6 mouse cells. The cells were incubated with a compound of the invention at various levels of oxygenation, then the cells were harvested and whole-cell protein preparations were made. Proteins from aerobic cells did not react, proteins from cells incubated at moderately low oxygen levels, 15 Torr, were slightly reactive, while proteins from cells incubated at very low oxygen levels were highly reactive. The closed triangle represent the relative reactivity of EF5 with cells containing 10 % oxygen; the open circles represent the relative reactivity of etanidazole in an anaerobic environment (nitrogen); the closed squares

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represent the relative activity of EF5 with cells containing 1% oxygen; and the closed circles represent the relative reactivity of EF5 in an anaerobic environment (nitrogen).

Figures 5-7 depict the immunohistochemical staining of various cells and tissues treated with a compound of the invention, then stained by mAbs of the invention. In Figures 5 and 6, a mAb of the invention was unmodified and a second antibody (goat anti-mouse-IgG1 labelled with the fluorescent dye fluorescein) was used to monitor the reaction. Figure 5 shows that tissue culture cells, 9L rat glioma, were stained much more heavily when incubated with a compound of the invention under hypoxic than under aerobic conditions. Figure 6 shows the same effect, except incubating a multicellular spheroid culture of mouse EMT6 cells with a compound of the invention. In this system, hypoxia is produced by metabolic depletion of oxygen by the outer cells leaving an inner annulus of hypoxic viable cells, and an inner core of anoxic, necrotic cells. The outer, unstained band represents viable oxygenated cells. Figure 7 shows that hypoxic cells *in vivo* can be analyzed in a similar manner after biopsy of a tumor specimen. In this case, EF5 was administered to a live tumor-bearing animal. A tissue section from a tumor biopsy was stained with a mAb of the invention which had been modified with the addition of a fluorescent dye (AMCA). The heavily staining, bright blue regions depict areas of high binding of the compound of the invention.

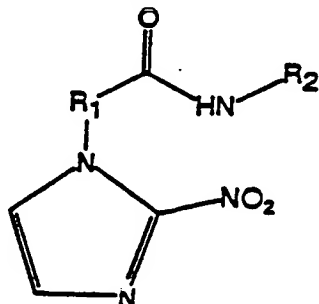
DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Hypoxic cells in solid tumors have been associated with treatment resistance by radiation, Moulder, *supra*, and some forms of chemotherapy, Kennedy, *supra*. Because of this effect, there is a great oncological need to identify hypoxic cells. In view of the foregoing, it is highly desirable to be able to assay for the presence of hypoxic cells in an animal or human tumor. However, there is no method currently available that can safely and consistently assay the oxygen

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level in mammalian tissue. The compounds and methods of the claimed invention address this need in the art.

The present invention provides a novel class of 2-nitroimidazole derivatives that are predictable oxygen
5 indicators using both immunohistochemical assays and imaging techniques, said compounds having the structure:



where R₁ is CH₂, CHX, or CX₂ where X is a halogen atom; and R₂ is C₁ to C₆ alkyl having up to about 6 halogen atoms.

The compounds of the invention are very useful in
10 detecting oxygen levels because of their dramatic specificity for hypoxic cells over normal, healthy, oxygenated tissue. Another aspect of the invention provides immunogenic conjugates comprising the novel compounds and a protein, and monoclonal antibodies specific for the novel compounds of the
15 invention, their protein conjugates, reductive byproducts, and adducts formed between mammalian tissue proteins and the compounds of the invention. Methods for detecting tissue hypoxia are also provided.

When hypoxic cells and aerobic cells are incubated
20 in the presence of compounds of the invention, the monoclonal antibodies of the invention selectively bind to hypoxic cells to a great degree. See Figures 1, 5-7. This preferential binding provides the basis for assaying tissue hypoxia in mammals.

25 Compounds of the invention can be synthesized using various reaction conditions depending on the starting material and ultimate requirements. For example, making of PET isotope-containing derivatives requires rapid addition of the F¹⁸ moiety followed by immediate purification and use
30 because of the short half-life of F¹⁸, 103 minutes.

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Generally, the compounds of the invention are synthesized under the following reaction conditions. The reaction may be performed in anhydrous tetrahydrofuran (THF) under argon and in the presence of iso-butylchloroformate and N-methylmorpholine. The starting material may be an alcohol derivative of 2-(2-nitro-1H-imidazol-1-yl) acetamide. The acetamide undergoes alkaline hydrolysis at the carbonyl group to yield an acid derivative of the 2-(2-nitro-1H-imidazol-1-yl). This is followed by nucleophilic substitution of a halogenated alkylamine at the acid intermediate's carbonyl group, to yield a halogenated nitroimidazole acetamide.

Alternatively, the starting material may be a 2-nitroimidazole acid derivative, which eliminates the need for the basic hydrolysis. The acid derivative then undergoes nucleophilic substitution with a halogenated alkylamine at the acid's carbonyl group to yield a halogenated nitroimidazole acetamide. Other synthetic methods will be apparent to those skilled in the art.

The reaction may yield a reaction slurry from which the product must be recovered. Methods of recovering the sample include any filtration or separation techniques known in the art. Such methods include, but are not limited to, vacuum filtration, separatory extraction, or distillation. A preferred method is filtration using air or liquid, but other methods will be apparent to those skilled in the art.

The filtration solid may further require washing with organic solvents to separate out impurities or other reaction intermediates or byproducts. Organic solvents include, but are not limited to, ether, methanol, ethanol, ethyl acetate, or hexanes. Ether is a preferred solvent, but other types of solvents will be apparent to those skilled in the art. Any organic solvent should be evaporated using methods known in the art. Evaporation methods may be accomplished at room temperature, by vacuum, aspiration, or by using latent heat. The evaporation methods are not

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limited to these techniques and other techniques will be apparent to those skilled in the art.

The reaction product is then purified using purification techniques known in the art. These techniques include, but are not limited to, column chromatography, flash chromatography, recrystallization, or gel chromatography. When using chromatographic purification methods, gradient elution is preferred. Combinations of organic solvents include, but are not limited to, methanol, acetonitrile, hexanes, carbontrichloride, and ethyl acetate. Other purification methods will be apparent to those skilled in the art.

In certain embodiments, the halogen atom of the compounds and compositions will be fluorine. In a more preferred embodiment, the compounds and compositions of the invention will have up to about 6 fluorine atoms. In accordance with other preferred embodiments, R_1 will be CH_2 . In another preferred embodiment, R_2 will be an alkyl group containing about 1 to 6 carbons and will have up to about 6 halogen atoms, preferably fluorine. In a more preferred embodiment of the invention, R_1 will be CH_2 and R_2 will be $CH_2CF_2CF_3$. This compound is referred to as EF5 throughout the application.

This invention is further directed to drug-protein conjugates formed between a compound of the invention and a suitable carrier protein, these compositions are referred to as antigens throughout this application. Antigens prepared using technology known in the art did not produce active mAbs, so previous procedures were substantially modified.

The prior art relates that antigen-forming reactions may be carried out between pH 4 to 7. It has now been found that these conditions fail to produce a sufficient number of drug-protein conjugates. It is greatly preferred to carry out the antigen-forming reactions at neutral or higher pH, preferably near neutrality. Under these conditions the drug-protein conjugation is much more efficient.

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The conjugation process is also much more efficient when the carrier protein contains cysteine sulfhydryl groups (PSH). Unfortunately, the cysteine residues of most proteins are a) few in number (e.g., hemocyanin); b) are not
5 accessible (e.g., alcohol dehydrogenase); or c) are oxidized as cystine dimers which do not bind reduced nitroaromatics. Although cystine dimers of several proteins can be very efficiently reduced via a radiochemical chain reaction, Koch & Raleigh, *Arch. Biochem. Biophys.*, 1991, 287, 75, the
10 resulting modified protein is often insoluble possibly because of the formation of disulfide bridges between molecules. It was not possible to reduce the protein cystines by addition of excess quantities of agents such as dithiothreitol or mercaptoethanol, which can simultaneously
15 reduce and stabilize cystine-containing proteins, because then adducts would preferentially form with the excess low-molecular weight thiol. Thus it was convenient to identify a protein with high cystine content, and having relative freedom from precipitation on radiochemical reduction. Bowman
20 Birk Inhibitor, a trypsin/chymotrypsin inhibitor from soybeans, (Bowman Birk Inhibitor (BBI) - 7 cystine bridges, molecular mass 7800) was found to have near optimal characteristics from this point of view, and reduction of up to an average of 8 cysteine residues was possible. The EF5-
25 BBI conjugates were then made in a second radiochemical reduction step.

Oxygen is excluded from the solutions using techniques previously described in Koch & Raleigh, *Arch. Biochem. Biophys.*, *supra*. Glass containers with specially
30 constructed ceramic-enclosed spin bars to eliminate oxygen released from teflon, Franko, et al., "Recent Results in Cancer Res. 95" in *Culture of Cellular Spheroids* 62 (Verlag 1984), were placed into leakproof aluminum chambers, and the oxygen-containing air was replaced by nitrogen using a number
35 of gas exchanges.

Suitable protein carriers include, but are not limited to, albumin (ALB), lysozyme (LYZ), and Bowman Birk

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inhibitor (BBI). In a more preferred embodiment of the immunogenic conjugate, the drug is EF5 and the protein is either BBI or LYZ.

This invention is also directed to monoclonal
5 antibodies that are highly specific for the compounds of this invention, their protein conjugates, reductive byproducts, and adducts formed between mammalian tissue proteins and the compounds of the invention. The drug-protein conjugate prepared according to the aforementioned procedure is used to
10 elicit antibody formation. When a drug-protein conjugate of the invention is bound to a protein carrier *in vitro* and administered to a mammal, monoclonal antibodies specific for compounds of the invention, their protein conjugates, reductive byproducts, and adducts formed between mammalian
15 hypoxic cells and the compounds of the invention can be raised. The preparation of monoclonal antibodies is known in the art. Particularly, Kohler and Milstein's method, Kohler, et al., *Nature*, 1975, 256, 495, with modifications as described in Knauf, et al., *Cancer Immunol. Immunotherapy*,
20 1986, 21, 217-225.

Generally, the drug-protein conjugate compositions were used to immunize mice using conventional techniques. See generally Knauf, et al., *supra*. A host is injected with a drug-protein conjugate of the invention, serving as antigen
25 to elicit an immune response. After an appropriate incubation period, blood is drained from the host and analyzed. When it was found that the host's serum showed strong activity against the antigen, the animal was sacrificed and its spleen cells used to make hybridoma
30 clones.

There are well-known methods to raise such antibodies. Thus, the host's spleen can be removed and the immune cells fused with appropriate myeloma cells to form a hybridoma using technology known in the art. Kohler, et al.,
35 *supra*. Such hybridomas are capable of producing monoclonal antibodies specific for the drug of the particular drug-protein conjugate administered to the mammal. Kohler, et al.,

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supra. In a preferred embodiment of the invention, the hybridoma clone will be conditioned to grow in serum-free medium. This ability to grow in serum-free medium permits facile purification of the antibodies and the easy addition
5 of detection moieties as a fluorophore, biotin, or an enzyme.

ELK-2 cells were conditioned to grow in serum free medium by slowly adapting them to lower and lower levels of serum, using a special medium formulation. See U.S. Patent No. 4,816,401, issued to Taupier & Lord, 1989.

10 The drug compounds of the invention are very useful in detecting oxygen levels because of their dramatic specificity for hypoxic cells over normal healthy oxygenated tissue. When hypoxic cells and aerobic cells are incubated in the presence of the new novel compounds, the monoclonal
15 antibodies of the invention selectively bind to hypoxic cells. This preferential binding provides the basis for assaying tissues in mammals.

For purposes of the current invention, mammals include, but are not limited to the Order Rodentia, such as
20 mice; Order Logomorpha, such as rabbits; more particularly the Order Carnivora, including Felines (cats) and Canines (dogs); even more particularly the Order Artiodactyla, Bovines (cows) and Suines (pigs); and the Order Perissodactyla, including Equines (horses); and most
25 particularly the Order Primates, Ceboids and Simoids (monkeys) and Anthropoids (humans and apes). The preferred mammals are humans.

Methods of detecting tissue hypoxia in mammalian tissue include, but are not limited to non-invasive imaging
30 techniques and immunohistochemistry. Imaging techniques include, but are not limited to single photon emission computed tomography (SPECT), PET, and nuclear magnetic resonance imaging, usually called magnetic resonance imaging (MRI). Generally, imaging techniques involve administering a
35 compound with marker atoms which can be detected externally to the mammal. A compound of the invention, is dissolved or dispersed in a pharmaceutically acceptable diluent, such as

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non-pyrogenic physiological saline, is administered to the mammal; time is allowing to clear non-metabolized compound; and tissue hypoxia is assayed using detectors of the marker atoms.

5 In a preferred embodiment of the invention, the compound will have at least 5 fluorine atoms. In a more preferred embodiment, of the invention R_1 will be CH_2 . In yet a more preferred embodiment, R_2 will be $CH_2CF_2CF_3$. In a still more preferred embodiment, R_1 will be CH_2 and R_2 will be
10 $CH_2CF_2CF_3$, yielding the compound EF5. Using PET the preferred isotope is F^{18} , using MRI the preferred isotope is F^{19} .

In another preferred embodiment of the invention, the detection techniques utilize the monoclonal antibodies
15 and immunohistochemistry. These techniques include, but are not limited to, immunoblotting or Western blotting, ELISA, sandwich assays, fluorescence, biotin or enzymatic labeling with or without secondary antibodies. Generally, immunohistochemistry involves staining cryosectioned tissue
20 samples. A mammal is injected with a compound of the invention dispersed or dissolved in a pharmaceutically accepted diluent. The compound selectively binds to the tissue proteins of hypoxic cells to form an adduct. A sample of tumor tissue is obtained and assayed using the invention's
25 monoclonal antibodies in conjunction with known immunohistological techniques. The degree of binding of the antibodies to the side chain of the adduct provides a measurement of the degree of hypoxia in the tumor tissue. See Figures 5-7. In a preferred embodiment of the invention,
30 the monoclonal antibodies of the invention can be used with cells or tissue sections fixed in paraformaldehyde. See Figures 6 and 7.

Methods of obtaining tissue samples for analysis, include any surgical and nonsurgical technique known in the
35 art. Surgical methods include, but are not limited to biopsy such as fine needle aspirate, core biopsy, dilation and curettage.

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Diagnostic kits are also within the scope of this invention. Such kits include monoclonal antibodies that can rapidly detect tissue hypoxia; and include a compound of the invention, individual or mixed monoclonal antibodies against
5 adducts formed between a compound of the invention and tissue proteins. Preferably, standards of manufactured protein adducts to be used as calibration sources for the assays are also included.

Preferred aspects of the invention are discussed in
10 the following examples. While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the invention is limited only in accordance with the appended claims.

EXAMPLE 1

15 Synthesis of EF5

EF5, 2(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide, was synthesized using 2-(2-nitro-1H-imidazol-1-yl)acetic acid as starting material. Nucleophilic substitution of a halogenated alkylamine
20 (2,2,3,3,3-pentafluoropropylamine hydrochloride) at the acid's carbonyl group was effected in anhydrous tetrahydrofuran (THF) under argon in the presence of isobutylchloroformate and N-methylmorpholine. Redistilled N-methylmorpholine (128 μ l, 1.17 mmol) was added to 2-(2-
25 nitro-1H-imidazol-1-yl)acetic acid (200mg, 1.17mmol) dissolved in anhydrous THF (20cc) under argon at 0°C followed 10 minutes later by fresh isobutylchloroformate (167 μ l, 1.287 mmol). After 30 minutes at 0°C, a preformed solution of 2,2,3,3,3-pentafluoropropylamine hydrochloride (237 mg, 1.287
30 mmol) and redistilled N-methylmorpholine (142 μ l, 1.287 mmol) in anhydrous THF (2 cc) was added to the creamy solution and the mixture stirred at room temperature for 3 hours. The reaction mixture was then filtered, the solid residue washed with ether and the combined organic solvents evaporated to
35 give a pale yellow solid. Purification by column chromatography (silica gel, 3% MeOH/CHCl₃) gave 2(2-nitro-1H-

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imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide as a white crystalline solid which recrystallized from EtOAc/hexanes, mp=136-137°C.

EXAMPLE 2

5 Antigen Production

Antigens were formed by radiation-induced covalent binding of EF5 to a protein carrier in an oxygen free environment. Oxygen was excluded from the solutions using techniques previously described in Koch & Raleigh, 1991, 10 *supra*. Glass containers with specially constructed ceramic-enclosed spin bars to eliminate oxygen released from teflon, Franko, et al., 1984, *supra*, were placed into leakproof aluminum chambers, and the oxygen-containing air was replaced by nitrogen using a series of gas exchanges. The time for 15 oxygen removal was measured using a polarographic oxygen sensor, as discussed in U.S. Patent No. 5,030,036 issued to Koch in 1991, enclosed in a nitrogen-flushed container of similar geometry to those used inside the chambers. The chambers were placed above stirring plates (Wheaton Biostir 20 4) during the oxygen removal process which took about three hours at a stirring rate of 4-5 Hz. Antigen formation was accomplished in a multistep process, each step consisting of a radiochemical reduction of protein disulfides in the absence of EF5, then addition of EF5 followed by a 25 radiochemical induction of adduct formation between EF5 and the reduced proteins. All irradiations were performed under anaerobic conditions, since even traces of oxygen inhibit the necessary reactions. The hypoxic solutions were irradiated in a Shepherd irradiator (Mark 1, Model 30) at a dose rate of 30 approximately 10 Gy per minute at room temperature.

High efficiencies of adduct formation are obtained only at substantially neutral pH. The EF5 was reductively bound to a protein in a solution containing 25-100 mM ammonium formate and 5-10 mM phosphate, pH 7.5. The formate 35 serves to convert oxidizing radicals from the radiation, which can interfere with the desired reduction reactions,

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into carbon dioxide anion radicals which are also reducing in character. Several proteins, each containing relatively large mole-fractions of cystine were considered: albumin (ALB), molecular mass 67000, 17 cystine cross links; lysozyme (LYZ), molecular mass 14000, 4 cystine cross links; Bowman Birk inhibitor (BBI), molecular mass 7800, 7 cystine cross links and RNAase, molecular mass 14000, 4 cystine crosslinks. The preferred protein was found to be BBI.

A typical cycle involved deoxygenating the protein (2 mg/cc, in 50 mM ammonium formate, 2.5 mM potassium phosphate), reducing protein cystine cross links by irradiating under hypoxia with sufficient dose to reduce about 50% of the cystine residues (typically 20-50 Gy), opening chambers and adding EF5 (150 micromolar), a second deoxygenation followed by sufficient radiation dose to reduce the drug (850 Gy), and ending with a purification/protein concentration step (centrifuge at 5000 G with molecular filter - Centricon 2). Overall recovery of initial protein was 30-50%. A small amount of radioactive EF5 (C^{14} -labelled in 2 position of nitroimidazole; specific activity 70 microcurie/mg) was included to keep track of the fraction of drug bound (initial specific activity of radioactivity in protein-containing solution minus specific activity of centricon filtrate).

After 3-5 cycles, the final protein concentration was determined (Pierce BCA assay). The specific activity of adducts was determined by precipitating a small amount of protein with 5% TCA, then dissolving in base. Following the aforementioned procedure results in about 1 conjugate per 3-4 lysozyme molecules, 1-3 BBI molecules, and 0.2-0.5 albumin molecules.

EXAMPLE 3

Preparation of Monoclonal Antibodies

Monoclonal antibodies are prepared by injecting intramuscularly two C57Br/cdJ x SJL/Br (H-2k) mice with 0.05 cc of the antigen at each of two sites, (300 μ g protein/cc;

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antigen conjugate to protein ratio was 0.5:1) emulsified with an equal volume of Freund's complete adjuvant. A booster injection of 0.1 ml antigen in Freund's incomplete adjuvant was given i.p. two months later. Three weeks after the
5 booster injection, a blood sample was taken, allowed to clot and the serum removed and tested using an ELISA assay.

The serum was characterized for its ability to react with the nitroimidazole compound using an ELISA assay technique. Protein or EF5-conjugated protein was allowed to
10 adhere to 96-well an ELISA plates (50 ng protein/well) and additional binding sites subsequently blocked with BLOTTO-Tween® (5% nonfat milk, 0.2% Tween-20) for 60 minutes at 37°C. Antisera dilutions were added and incubated for 45 minutes at 37°C, unbound antibody was removed by washing 3x
15 with 0.02% Tween-20 in PBS and 2x with PBS. To each well was added 0.1 cc of biotinylated horse anti-mouse IgG1 diluted in PBS and incubated for 45 minutes at 37°C, then washed as above. An avidin-biotin peroxidase complex (Vectastain ABC Reagent) diluted in PBS containing 0.1% Tween 20 was added
20 and incubated for 45 minutes at 37°C. The wells were then washed 5x as above. Each well then received 0.05 cc of horseradish peroxidase substrate (0.05 mg/cc OPD [o-phenylenediamine dihydrochloride] in 50 mM citrate/phosphate buffer, pH 4.5, 0.015% hydrogen peroxide). The color was
25 allowed to develop at room temperature for 5-30 minutes, then the reaction was stopped by adding 0.05 cc of 4N H₂SO₄. Finally, the optical density was recorded at 490 nm.

A mouse whose antiserum reacted with the EF5 whether it was conjugated to either the immunizing protein
30 (BBI) or other proteins (ALB, LYZ) was selected. This mouse received additional intraperitoneal injections of EF5-protein conjugate (80 µg) at four, three, two and one days before sacrifice and spleen removal. The fusion was performed as described in Harwell, et al., *J. Immunol. Methods*, 1984, 66,
35 59. The polyethylene glycol (PEG) used was Sigma #P7777 prepared at 35% in serum-free media/5% dimethylsulfoxide (DMSO) and the pH adjusted to 7.4 with 4N NaOH. All cells

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and reagents were warmed to 37°C prior to the fusion. After the fusion, the cells were bulk cultured in a roller bottle for two days. The cells were then centrifuged, resuspended in 10 cc of freezing medium (culture medium with 40% fetal
5 bovine serum -S and 10% DMSO) and frozen at 1 cc per vial.

One vial of the fusion was subsequently thawed and cultured in 5 each 96-well microliter plates in 10% FBS, HAT-containing culture medium (1.36 mg/cc hypoxanthine, 0.038 mg/cc aminopterin, 0.0176 mg/cc thymidine) to select for
10 fused cells, with 2000 irradiated mouse peritoneal cells per well as feeder cells. Wells were screened for specific antibody-producing cells using the ELISA assay described above and BBI and BBI-F5E conjugate as antigens. Six
15 hybridomas were selected based on the differentiation of these two antigens, and were further screened against a panel of additional antigens. One of these, ELK-2-4,3,4 (ELK-2), was selected based on its strong selective reaction against protein-EF5 conjugates and lack of reactivity against any
20 other antigens tested, including whole cell protein preparations. See Table 1 (row 1) and Figures 2-4.

TABLE 1

ELISA Assay: 10% Portion of 3rd Fusion (Anti EF5-Lysozyme)
vs ELK-2 (Anti EF5-BBI)

Hybridoma	BSA	BSA-EF5	Lys	Lys-EF5	BBI	BBI-EF5	9L/Control	9L-EF5	EMT6/Control	EMT6-EF5
ELK 2-4,3,4	0	>2	.01	.18	0	>2	0	>2	.01	1.02
ELK 3-5.17	0	>2	.01	1.26	0	>2	0	0.44	.02	0.26
ELK 3-23	0	>2	0	.86	0	>2	.03	0.41	.04	0.25
ELK 3-29	0	>2	0	0.20	0	>2	0	0.05	0	0.03
ELK 3-2.9	.01	.01	>2	>2	0	0.01	0	0	0	0
3-40	.01	>2	>2	>2	0	>2	.01	0.05	.02	.05
3-41	0	>2	0	0.01	0	1.02	0	0.07	.01	.04
3-42	0	>2	>2	>2	0	>2	0	0.13	0	.08
3-43	0	>2	>2	>2	0	>2	0.01	0.70	.01	.44
3-44	0	>2	>2	>2	0	>2	0.01	0.14	.02	.10
3-45	0	>2	>2	>2	0	>2	0.01	0.07	.02	.08
3-46	0.01	0.65	>2	>2	.01	0.59	0	0.02	.01	.03
3-47	0	0.13	.02	0.07	0	0.30	0	0.01	0	.02
3-48	0	>2	>2	>2	0	>2	0	0.02	0	.01
3-49	0.01	0.12	>2	>2	0.01	0.17	0.04	0.03	0.05	.05
3-50	0	>2	0	0.56	0	>2	0	0.16	0.01	0.11
3-51	0	>2	0.01	0.12	0	>2	0.01	1.86	0.02	1.60
3-52	0	0.05	0.01	1.68	0	0.03	0	0.03	0	.02
3-53	0	>2	1.33	>2	0	>2	0.01	0.05	0	.04
3-54	0.01	>2	0	0.14	0	>2	0	0.05	0	.04
3-550	0	>2	0.07	0.28	0	>2	0	0.04	0	.04
3-56	0	>2	0	0.25	0	>2	0	0.07	0	0.05

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MAbs from the most differentially specific clone, designated as ELK-2, were then characterized. Although MAbs from ELK-2 reacted strongly with the parent 2-nitroimidazole, almost no reactivity was found with other nitroheterocyclics. For example, no measurable reactivity was observed for etanidazole at 1000 fold higher concentrations; etanidazole differs from EF5 only in the absence of fluorines on the side chain (see Figure 2).

Another mouse, which had been immunized with a compound of the invention adducted to lysozyme was treated in a similar manner. Table 1 depicts the responses of the various clones to a large variety of proteins, each with versus without EF5 adducts. The initial screens of the clones were made based on a positive response to albumin-EF5, but a negative response to albumin alone. Of the sixteen clones examined, almost all possibilities of response were noted against the alternate antigens, but only two clones, #43 and #51, showed a strong differentially positive response against whole cell protein preparations from hypoxic cells incubated with EF5. Table 1 shows the importance of a wide variety of test antigens, the most important being whole cell protein digests, and that the techniques described can reliably produce mAb-producing hybridomas with high specificity to the desired antigens.

EXAMPLE 4

Binding of EF5 to Cells in Tissue Culture Under Defined Oxygen Conditions.

Cells were thawed from frozen stock on a roughly semi-annual basis and tests were made routinely to ensure that the cultures were free from mycoplasma and other contaminations. The cells were cultured (37°C, 95% air + 5% carbon dioxide, 100% relative humidity) in the exponential phase of growth by twice weekly transfers using Eagle's Minimal Essential medium containing 12.5% v/v fetal calf serum and antibiotics (all culture solutions from GIBCO). On the day before an experiment, cells were trypsinized and plated onto glass Petri dishes - approximately 200,000 cells confined to the central

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area of the dish followed by overnight incubation at 37°C as described previously. Koch, *Radiat. Res.*, 1984, 97, 434.

The dishes were then removed from the incubator, cooled to 0°C, and their medium replaced with drug-containing medium, first as a rinse (1 ml) which was simply aspirated and then as the actual medium used for the experiment (1 ml). Dishes were placed in leak-proof aluminum chambers which were connected to a manifold allowing them to be deoxygenated with a series of gas exchanges taking approximately thirty minutes. The confinement of cells to the central area of the dish, and the use of a small volume of medium allows very rapid equilibration of the gas and liquid phase to improve the control of oxygen concentration. Koch, 1984, *supra*. After deoxygenation, the chambers were incubated at 37°C. To prevent minor gradients of oxygen or potentially larger gradients of nutrients/metabolites, the chambers were also shaken gently (1 Hz, 2.5 cm stroke). To assay binding of radioactive nitroheterocyclics after incubation under defined experimental conditions, Koch, 1984, *supra*., the chambers were removed from the incubator and opened (allowing immediate reoxygenation), and the dishes were cooled on ice. The radioactive medium was removed. Two rinses with HEPES buffered Earle's balanced salt solution (EBSS) were followed by the addition of fresh medium and the dishes were incubated at 37°C for 15 minutes. Another rinse in EBSS was followed by cell removal with 0.05% trypsin (40:1 dilution of SIGMA '40X') in calcium and magnesium free EBSS. An equal volume of serum-containing medium was added to stop the trypsin and a portion of the cell suspension was counted.

The cells were concentrated by centrifugation at 1000 rpm for ten minutes followed by resuspension in 200 µl of EBSS, on ice, and then the cells were disrupted by the addition of 100 microliter of 1 M ice-cold trichloroacetic acid (TCA). The TCA precipitate was concentrated by centrifugation at 2500 rpm for 20 minutes. The TCA supernatant was saved and the pellet was dissolved in 0.2 ml of 1N NaOH, followed by neutralization with acetic acid. The clear TCA supernatant and the dissolved

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pellet were added with scintillation fluid to vials and counted in a Beckman liquid scintillation counter (LSC), resulting in a separation of acid soluble and acid precipitable counts. The above procedure results in background levels of incorporated radioactivity when radioactive sensitizers were incubated at low temperatures (4°C) in hypoxia, or at 37°C in air. Since the cell number, drug concentration and specific activity, and LSC efficiency were all known, the absolute incorporation of adducts could be calculated as picomoles per cell per hour of incubation at 37°C.

It can be seen in Figure 1 that binding occurs to a much greater extent in hypoxic cells (circles) than cells at intermediate oxygen levels (~3 Torr - squares) and almost no binding is seen for cells containing a higher level of oxygen (15 Torr-triangles). In each case the solid symbols are for TCA precipitable adducts, and the open symbols are for TCA soluble adducts. The cells in this experiment were 9L rat glioma cells.

EXAMPLE 5

Testing the mAbs' Specificity Using ELISA Techniques

Figure 2 shows that the antibody designated ELK-2 recognizes only a compound of the invention, EF5, with a variety of other nitroaromatic compounds showing at least 100 fold less reactivity toward the antibody. The compounds are represented by the following symbols: solid circles represent EF5; open circles represent ETAN; the open triangles represent MISO; closed squares represent CCI-103F; crosses represent NP-6; and open squares are DMM.

Figure 3 demonstrates that addition of the AMCA fluorescent moiety to a monoclonal antibody of the invention, using two different coupling chemistries does not reduce its high specificity for its target antigen, the EF5-BBI conjugate. The open circles represent the monoclonal antibodies' specificity without the presence of the detection moiety. The AMCA molecules were added using standard protein modification kits available from Pierce, which involves the coupling of the

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dye to an N-hydroxysulfosuccinimide group (NHSD sulfo Hi-closed triangles), which then reacts with primary amines of the protein of interest, or similar methods where the reactive group is a pyridyldithiopropionamide (HPDP hi-closed squares), which reacts with free thiol groups on the protein of interest. The thiols were generated using mild radiochemical reduction methods (See Example 1) to reduce a small portion of the antibody's disulfide residues.

Figure 4 represents an example of how a clinical assay could be performed. In this case, cells treated with EF5 under hypoxia were homogenized using standard protein extraction techniques. The resulting protein preparations were added to 96 well ELISA plates, as described above for Example 3, and analyzed using ELISA competition techniques. It can be seen that proteins from hypoxic cells were much more reactive than those from cells incubated at intermediate oxygen concentrations, and that proteins from aerobic cells were non-reactive. The closed triangle represent the relative reactivity of EF5 with cells containing 10 % oxygen; the open circles represent the relative reactivity of etanidazole in an anaerobic environment (nitrogen); the closed squares represent the relative activity of EF5 with cells containing 1% oxygen; and the closed circles represent the relative reactivity of EF5 in an anaerobic environment (nitrogen).

EXAMPLE 6

Imaging Techniques (Figures 5-7)

Various cells and tissues, either caused or expected to contain hypoxic cells, were incubated with a compound of the invention, EF5, and then stained using a mAb of the invention, ELK-2. The binding of the antibody was visualized in one of two ways. First, utilizing a fluorescein-labelled second antibody (Jackson Laboratories) recognizing the isotype (mouse IgG1) of the ELK-2 antibody. Second, utilizing ELK-2 which had been made autofluorescent via the addition of AMCA as described above.

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Figure 5 compares the binding of antibody to 9L glioma cells treated with 0.5 mM EF5 in the presence of either air, represented by the dark region in the photomicrograph, versus nitrogen, represented by the light region on the photomicrograph. The exposures of drugs, antibodies, and photography were all identical in the two halves of the figure. Only the oxygen concentration during incubation was varied. The cells were allowed to attach to microscope slides, then they were air dried, and briefly exposed to a 0.1% solution of triton X-100, followed by rinsing to remove the detergent. The slides were treated overnight at 4°C with ELK-2 antibody and rinsed for several minutes in PBS. They were then stained with fluorescein-labelled goat anti-mouse IgG1, rinsed to remove unbound second antibody, and observed using a fluorescent microscope.

Figure 6 shows the results of a similar experiment where whole spheroids growing in spinner flasks were incubated with 0.5 mM EF5 for 4 hours. The spheroids were removed, washed, embedded in OCT compound and frozen on dry ice. Cryostat sections (10 micron thickness) were cut, placed on poly-L-lysine coated glass slides, and the sections fixed in 1% paraformaldehyde for 10 minutes. After rinsing, blocking antisera (2% normal goat serum) was added and incubated for 30 minutes at room temperature. The slides were treated with ELK-2 overnight at 4°C, and were then washed 3 times, then treated with a fluorescein-labelled second antibody as indicated above. The slides were then washed and observed with a fluorescent microscope. The photomicrograph shows that the outer rim of the spheroid is completely unstained, but that the staining intensity increases dramatically for cells in the spheroid interior which are expected to be hypoxic.

Figure 7 represents another example, using an actual tumor bearing animal. A BALB/c mouse bearing an EMT6 tumor was injected intraperitoneally with 1.2 cc of 2 mM EF5. Forty-eight hours later, the mouse was sacrificed, the tumor removed, embedded in OCT compound and frozen on dry ice. Cryostat sections of about 20 microns thickness were cut, and the

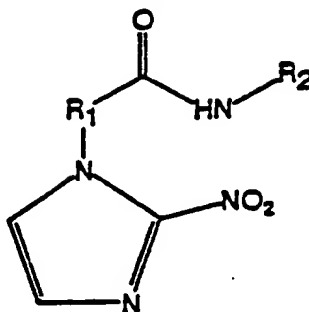
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sections treated as above for the spheroid sections, except that only one antibody was used, namely the AMCA conjugated ELK-2 described above and in Figure 4. The AMCA dye fluoresces in the blue, using UV excitation. The photomicrograph's bright areas are those expected to contain hypoxic cells. Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

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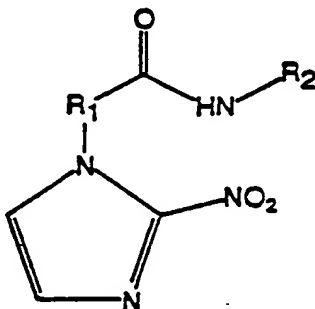
What is claimed is

1. A compound having the formula



wherein R_1 is CH_2 , CHX , or CX_2 where X is a halogen atom; and R_2 is C_1 to C_6 alkyl having up to about 6 halogen atoms.

- 5 2. The compound of Claim 1 wherein the halogen atom is fluorine.
3. The compound of Claim 1 wherein R_1 is CH_2 .
4. The compound of Claim 1 wherein R_2 is $CH_2CF_2CF_3$.
- 10 5. The compound of Claim 1 wherein R_1 is CH_2 , and R_2 is $CH_2CF_2CF_3$.
6. The compound of Claim 1 comprising about five fluorine atoms.
7. The compound of Claim 1 dissolved or dispersed
15 in a pharmaceutically acceptable carrier or diluent.
8. A compound bound to a protein, the compound having the formula:



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wherein R_1 is CH_2 , CHX , or CX_2 where X is a halogen atom; and R_2 is C_1 to C_6 alkyl having up to about 6 halogen atoms.

9. The compound of Claim 8 wherein R_1 is CH_2 and R_2 is $CH_2CF_2CF_3$.

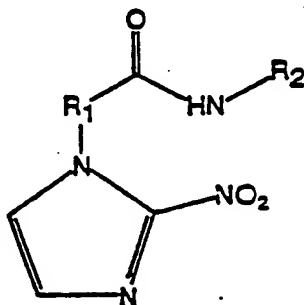
5 10. The compound of Claim 8 wherein the protein is albumin, lysozyme, or Bowman Birk inhibitor.

11. The compound of Claim 8 wherein the protein is Bowman Birk inhibitor.

12. The compound of Claim 8 wherein the protein is
10 lysozyme.

13. A method for preparing a monoclonal antibody comprising:

a) introducing into a mammal a compound bound to a protein, the compound having the formula:



15 wherein R_1 is CH_2 , CHX , or CX_2 where X is a halogen atom; and R_2 is C_1 to C_6 alkyl having up to about 6 halogen atoms; and

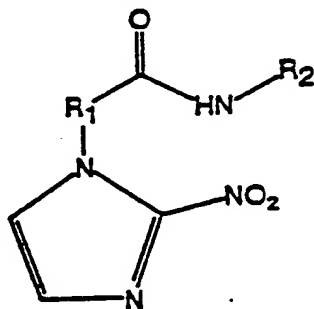
b) fusing immune cells of the mammal with mammalian myeloma cells forming a hybridoma that produces antibodies specific for the compound bound to the protein.

20 14. The method of Claim 13 wherein the halogen atom is fluorine.

15. The method of Claim 13 wherein R_1 is CH_2 .

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16. The method of Claim 13 wherein R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$.
17. The method of Claim 13 wherein R_1 is CH_2 and R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$.
18. The method of Claim 13 wherein the compound has
5 at least five fluorine atoms.
19. The method of Claim 13 wherein the protein is albumin, lysozyme, or Bowman Birk inhibitor.
20. The method of Claim 13 wherein R_1 is CH_2 , R_2 is $\text{H}_2\text{CF}_2\text{CF}_3$, and the protein is Bowman Birk inhibitor.
- 10 21. The method of Claim 13 wherein R_1 is CH_2 , R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$, and the protein is lysozyme.
22. A monoclonal antibody specific for a compound, the compound's protein conjugate, the compound's reductive byproduct, or adduct formed between the compound and tissue
15 protein, the compound having the formula:



- wherein R_1 is CH_2 , CHX , or CX_2 where X is a halogen atom; and R_2 is C_1 to C_6 alkyl having up to about 6 halogen atoms.
23. The monoclonal antibody of Claim 22 wherein the halogen atom is fluorine.
- 20 24. The monoclonal antibody of Claim 22 wherein R_1 is CH_2 .

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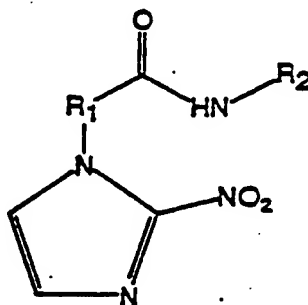
25. The monoclonal antibody of Claim 22 wherein R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$.

26. The monoclonal antibody of Claim 22 wherein R_1 is CH_2 and R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$.

5 27. The monoclonal antibody of Claim 22 wherein the compound has at least five fluorine atoms.

28. A method for detecting tissue hypoxia in a mammal comprising:

a) introducing into the mammal a compound
10 having the formula:



wherein R_1 is CH_2 , CHX , or CX_2 where X is a halogen atom; and R_2 is C_1 to C_6 alkyl having about 6 halogen atoms; and

b) imaging the portion of the mammal containing the tissue.

15 29. The method of Claim 28 wherein the detection technique is PET or MRI.

30. The method of Claim 28 wherein R_1 is CH_2 and R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$.

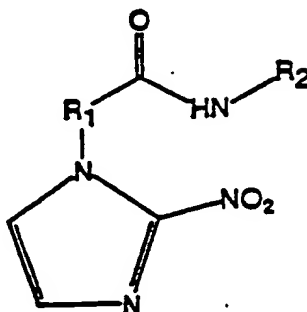
31. The method of Claim 28 wherein X is F^{18} and the
20 detection technique is PET.

32. The method of Claim 28 wherein X is F^{19} and the detection technique is MRI.

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33. A method for detecting tissue hypoxia in a mammal comprising:

a) introducing into the mammal a compound having the formula:



5 wherein R_1 is CH_2 , CHX , or CX_2 where X is a halogen atom; and R_2 is C_1 to C_6 alkyl having about 6 halogen atoms;

b) administering a labelled monoclonal antibody specific for the compound; and

10 c) imaging the portion of the mammal containing the tissue.

34. The method of Claim 33 wherein R_1 is CH_2 and R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$.

35. The method of Claim 33 wherein the imaging technique is PET or SPECT.

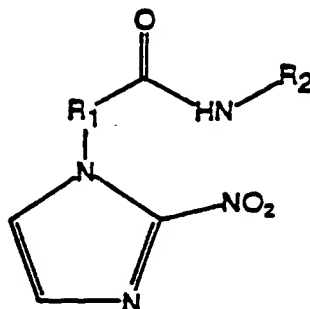
15 36. The method of Claim 33 wherein R_1 is CH_2 and R_2 is $\text{CH}_2\text{CF}_2\text{CF}_3$.

37. The method of Claim 33 wherein the label is a positron or gamma emitting isotope.

20 38. The method of Claim 33 wherein the imaging technique is MRI.

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39. A kit for detecting tissue hypoxia comprising a compound having the formula:



wherein R₁ is CH₂, CHX, or CX₂ where X is a halogen atom; and R₂ is C₁ to C₆ alkyl having up to about 6 halogen atoms; a protein;
5 and a monoclonal antibody specific for the compound, the compound's protein conjugate, the compound's reductive byproduct, or adduct formed between the compound and tissue proteins; standards comprising the compound bound to a protein; a monoclonal antibody bound to a detection moiety; and
10 detection moieties.

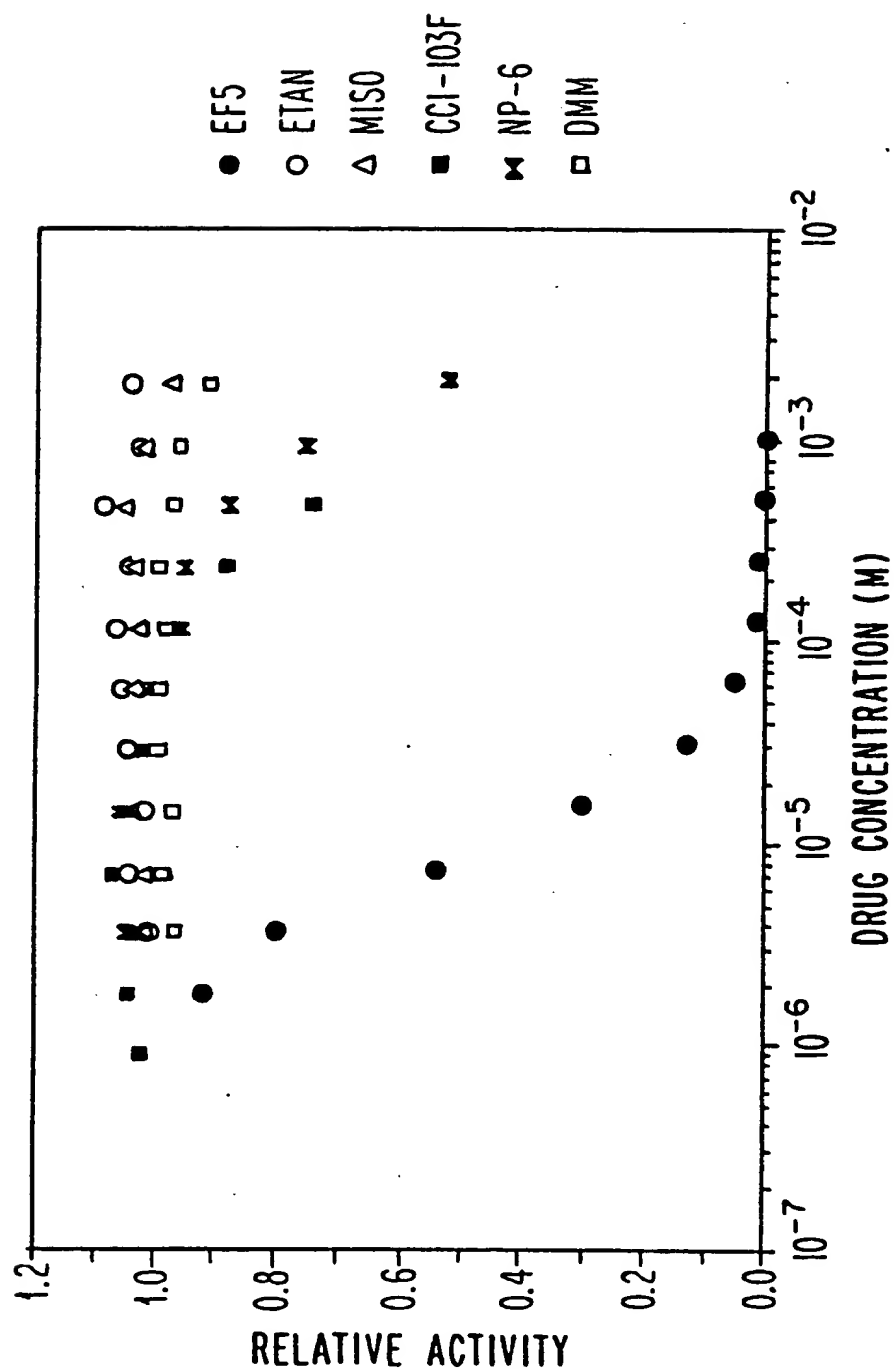
40. The kit of Claim 39 wherein the detection moiety is a fluorophore, biotin, radioactive isotopes, or an enzyme.

41. The kit of Claim 39 wherein R₁ is CH₂, R₂ is CH₂CF₂CF₃.

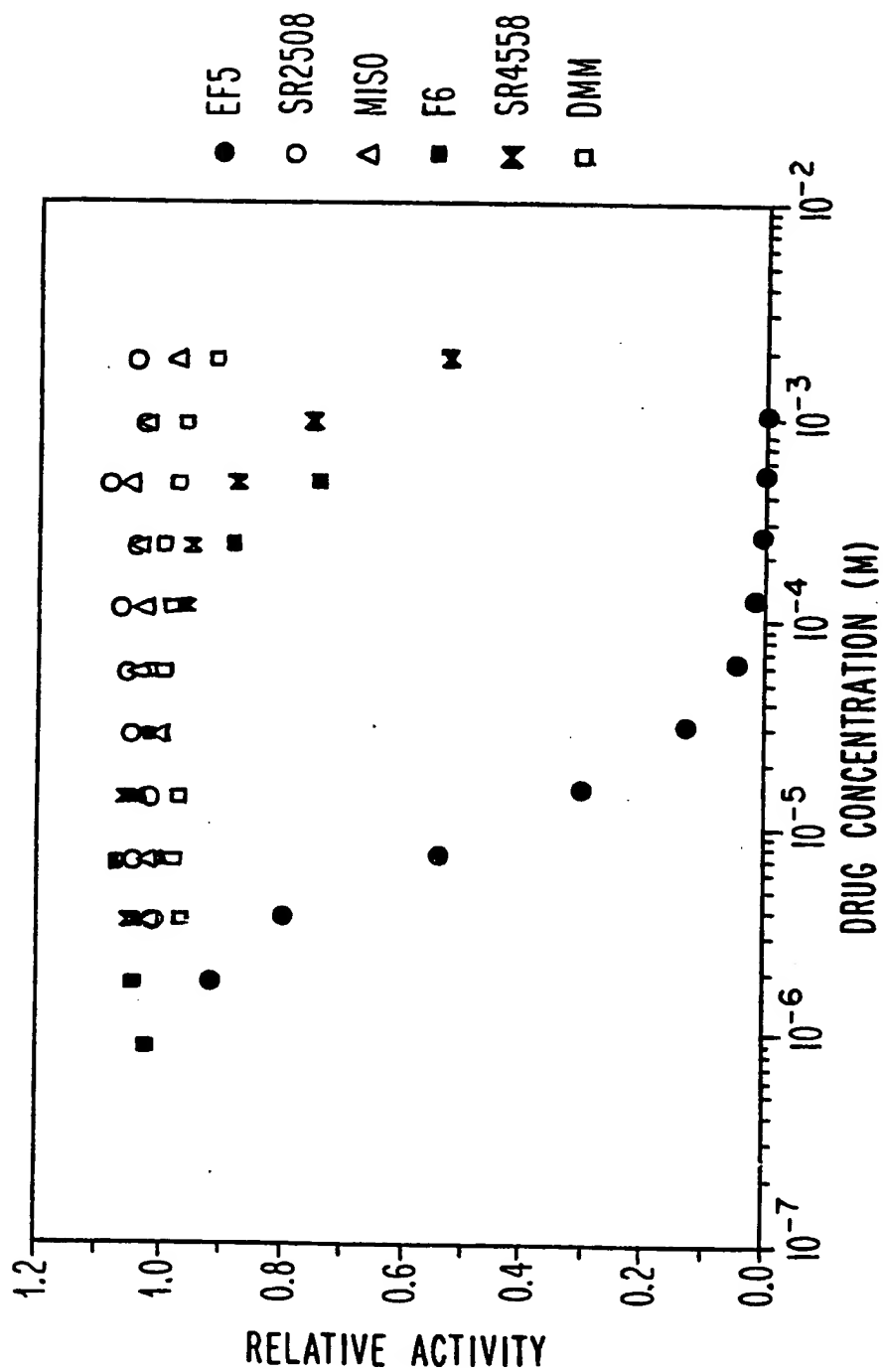
15 42. The kit of Claim 39 wherein the proteins of the standards include albumin, Bowman Birk inhibitor, or lysozyme.

43. The kit of Claim 39 wherein R₁ is CH₂, R₂ is CH₂CF₂CF₃, and the protein is Bowman Birk inhibitor.

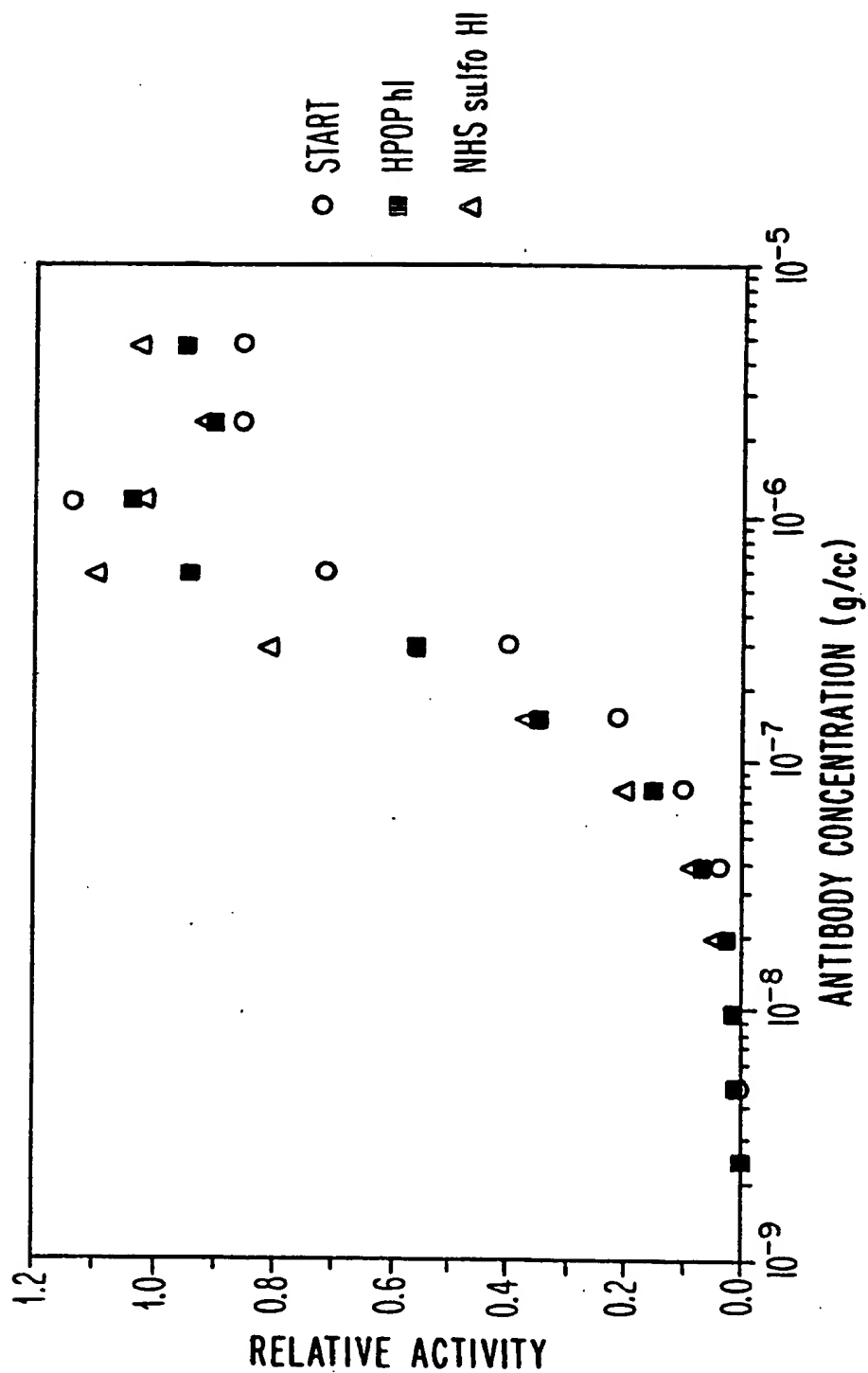
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*Fig. 1*

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*Fig. 2*

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***Fig. 3***

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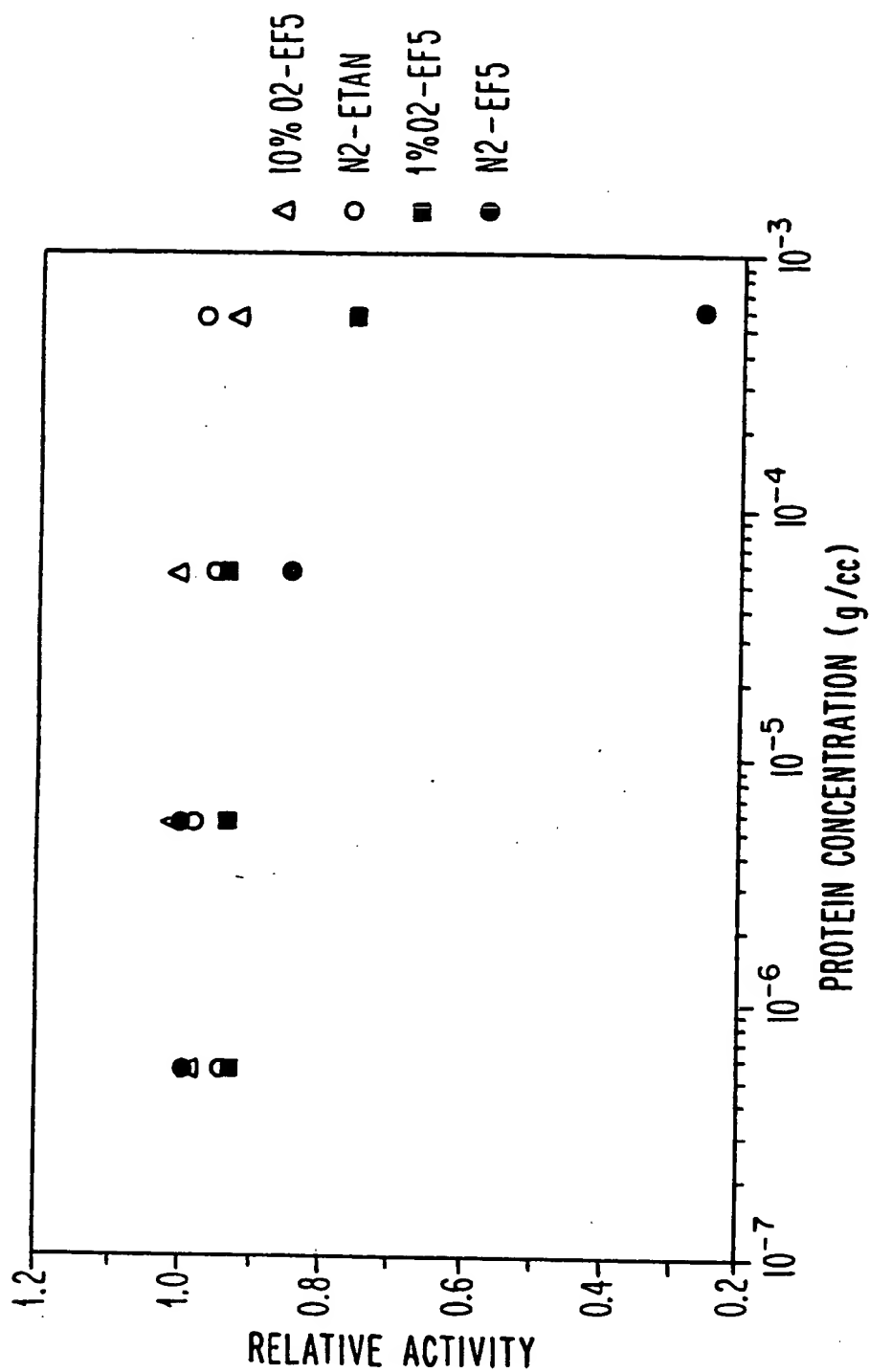


Fig. 4

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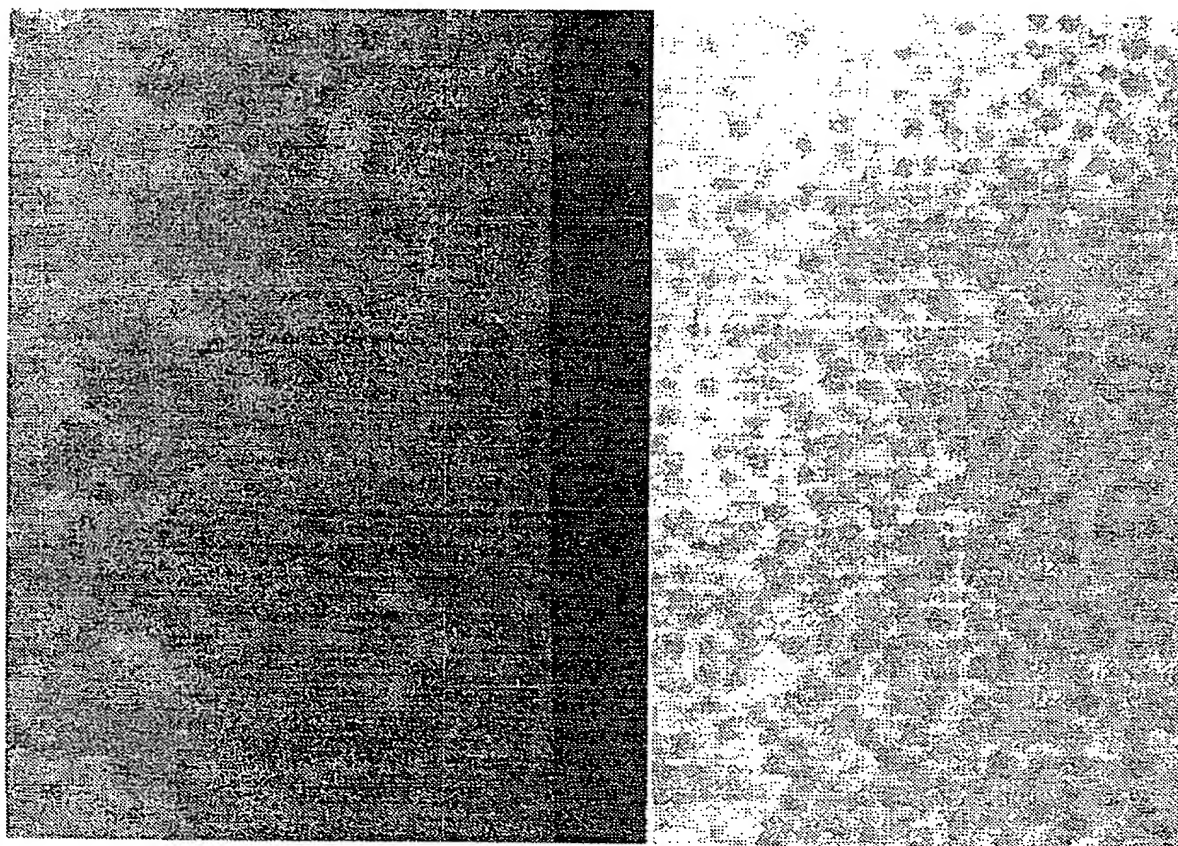


FIG. 5

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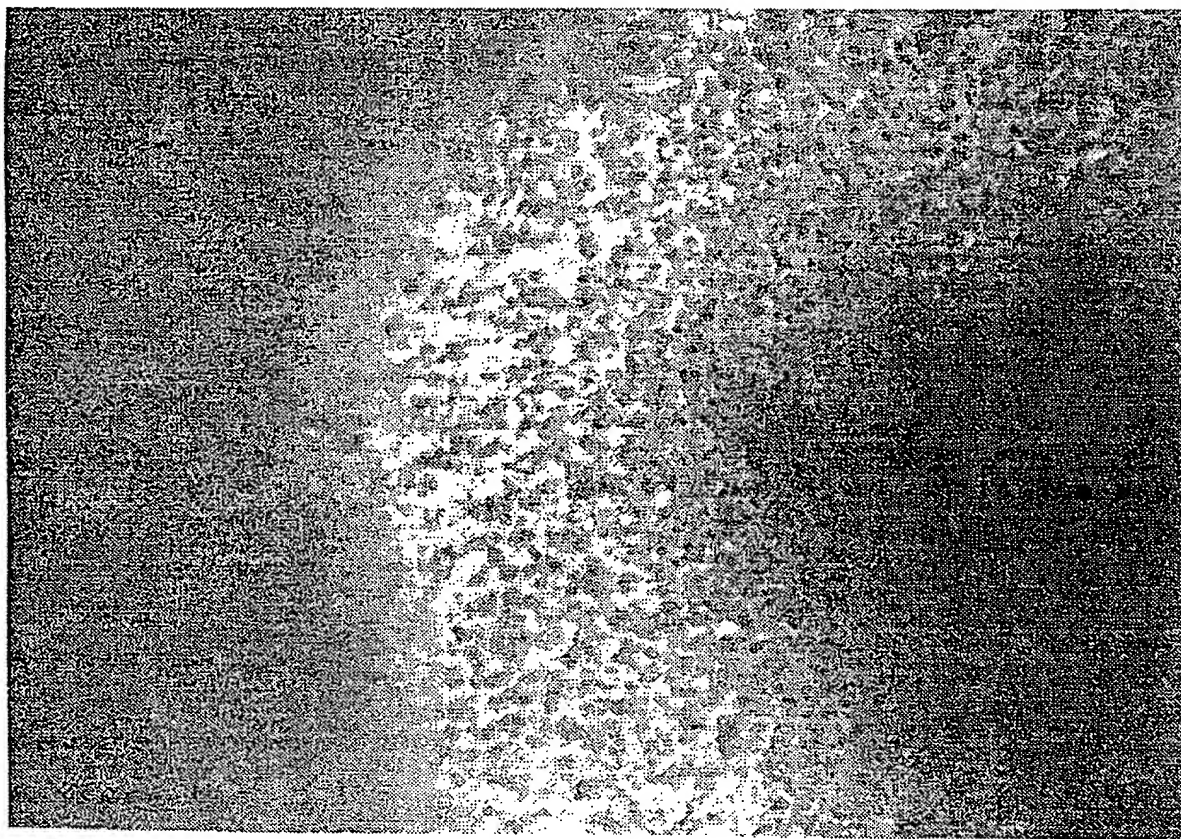


FIG. 6

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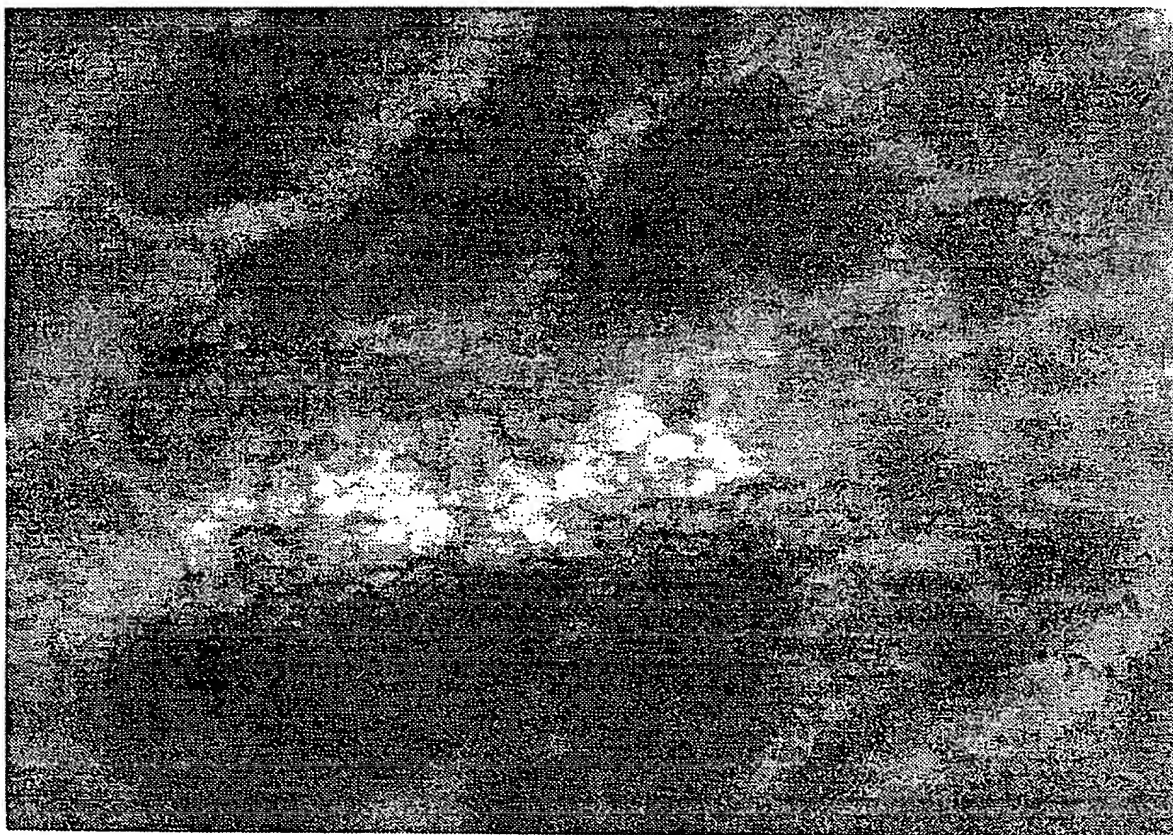


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11190

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/61; 424/1.11, 1.49, 9, 85.91; 435/188; 436/503; 530/388.9, 391.1, 405, 409; 548/327.5, 328.5, 338.1, 343.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
File Registry, File CA, File APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<input checked="" type="checkbox"/> X Y	US, A, 3,679,698 (BEAMAN et al) 25 July 1972, abstract and col. 1-2, 9-10.	<u>1,3,7</u> 1-43
<input checked="" type="checkbox"/> X Y	Chemical Abstracts, Volume 71, No. 5, issued 1969, Beaman et al, "Studies in the nitromidazole series. III. 2-nitro-imidazole derivatives substituted in the 1-position", see pages 324-325, abstract No. 22065t, Antimicrob. Agents Chemother. 1967 (pub. 1968), 520-530.	<u>1,3,7</u> 1-43

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be part of particular relevance	* X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

17 February 1994

Date of mailing of the international search report

MAR 11 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. not applicable

Authorized officer

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Telephone No. (703)308-0196

INTERNATIONAL SEARCH REPORT

Internal application No.

PCT/US93/11190

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Chemical Abstracts, Volume 70, No. 3, issued 1969, Grunberg et al, "Antiprotozoan and antibacterial activity of 2-nitroimidazole derivatives", see page 138, column 1, abstract No. 10175v, Antimicrob . Agents Chemother. 1967 (Pub. 1968), 513-19.	<u>1,3,7</u> 1-43
Y	US, A, 4,241,060 (SMITHEN) 23 December 1980, abstract.	1-43
Y	US, A, 5,086,068 (RALEIGH et al) 04 February 1992, entire patent.	1-43
A	Journal of Pharmaceutical Sciences, Volume 76, No. 5, issued May 1987, Heindel et al, "Macromolecular Attachment as a Metabolic Stabilizer for a Labile Radiosensitizer", pages 384-386.	

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C07D 233/66, 233/91, 235/04, 487/00; C07K 15/28, 17/02; C12N 9/96; A61K 39/385, 39/44, 43/00, 49/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

422/61; 424/1.11, 1.49, 9; 435/188; 436/503; 530/388.9, 405, 409; 548/327.5, 328.5, 338.1, 343.1

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claims 1-7, 28-32 and 39-43, drawn to 2-nitroimidazole derivatives of claim 1, method of using the product to detect hypoxia and kit claims comprising the product, classified in Class/subclass 548/327.5, 328.5, 338.1, 343.1; 424/1.11,9; 436/503; 422/61.

II. Claims 8-27 and 33-43, drawn to protein conjugated to the compound of claim 1, method of making monoclonal antibody (Mab) using the conjugates, Mab thereof, using the compound and the antibodies conjugated to a label to detect hypoxia and kits thereof, classified in Class/subclasses 435/188; 530/388.9, 405, 408; 424/1.49; 436/503; 422/61.